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Cytotoxic KLRG1 expressing lymphocytes invade portal tracts in primary biliary cholangitis

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ABSTRACT

Primary biliary cholangitis (PBC) is a chronic autoimmune liver disease with an immunopathogenesis that includes highly differentiated cytotoxic T cell infiltration in portal areas. We have taken advantage of a large and well-defined cohort of patients with PBC, AIH, chronic hepatitis virus, and healthy controls to study for the presence of highly differentiated T cells which express the killer cell lectin-like receptor G1 (KLRG1). Such studies were performed using both liver and peripheral blood mononuclear cells. In particular, gene expression data (GSE79850) from 16 PBC patients stratified according to future risk of liver transplantation were analyzed for markers of highly differentiated cytotoxic T cells. Liver biopsy samples from 44 PBC patients were studied by immunohistochemistry and a separate cohort of PBC blood samples were studied by flow cytometry. Gene expression data demonstrated correlation of increased *KLRG1* and cytotoxic lymphocyte molecules, such as granzyme B (*GZMB*) and perforin (*PRF1*), to disease severity as measured by future risk of liver transplantation. Immunohistochemistry demonstrated abundant infiltration of KLRG1+ cells into liver portal areas (mean of 45% of infiltrating cells, range 25–75%) positively correlated with hepatic inflammatory ($r = 0.47$, $p = 0.001$) and hepatic fibrosis ($r = 0.34$, $p = 0.021$) scores. KLRG1+ lymphocyte liver portal area infiltration was positively correlated with serum alkaline phosphatase ($r = 0.45$, $p = 0.005$) and GGT ($r = 0.40$, $p = 0.014$), and AST ($r = 0.35$, $p = 0.033$) levels. Mononuclear blood flow cytometry studies showed KLRG1+ lymphocytes had greater levels of cytotoxic molecules (granzyme B and perforin), inflammatory cytokines (IFN-γ and TNF-α) and inflammatory chemokine receptors (CCR5 and CX3CR1) than KLRG1-counterparts. However, clearly the most significant data was that found in liver with the intense portal infiltrates that are unique to PBC. *Conclusion*: Highly cytotoxic KLRG1+ lymphocytes have invaded PBC liver portal areas. Liver KLRG1 gene expression and

Abbreviations: KLRG1, killer cell lectin-like receptor G1; PBC, primary biliary cholangitis; AIH, autoimmune hepatitis; CHB, chronic hepatitis virus B; HC, healthy controls; GZMB, granzyme B; PRF1, perforin; TEM, T effector memory cells; TEMRA, T effector memory RA cells; BEC, biliary epithelial cells; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha; ALP, alkaline phosphatase; GGT, γ-glutamyl transferase; ALT, alanine transaminase; AST, aspartate aminotransferase; IgM, immunoglobulin M; IQR, interquartile range; TBIL, total bilirubin; IgG, immunoglobulin G; AMA, anti-mitochondrial antibody

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the abundance of KLRG1+ lymphocytes are positively correlated with disease biomarkers used as clinical trial outcome measures (liver transplantation and serum alkaline phosphatase), suggesting the targeting of KLRG1+ lymphocytes as a rational approach for PBC therapeutic drug development.

1. Introduction

Primary biliary cholangitis (PBC) is a chronic autoimmune liver disease characterized by progressive destruction of intrahepatic bile ducts, resulting in cholestasis and bile acid toxicity [1,2]. It is primarily a disease of middle-aged women, with prevalence as high as 402 per million [3,4]. Disease progression is associated with chronic cholestasis producing fatigue, pruritus, dyslipidemia, fat-soluble vitamin deficiency with consequent bone loss, portal hypertensive-related complications including gastroesophageal varices, and eventual liver fibrosis and need for transplantation. The introduction of UDCA has significant improved the clinical outcomes of PBC, however, about one third PBC patients do not respond adequately. So far, in PBC patients, the rate of progression of cirrhosis is 13% in a follow-up period of 6 years, the transplantation-free survival rates is 78% at 10 years, and the overall liver transplantation rate is 9% [5–7].

PBC liver demonstrates infiltration of T cells into the portal tract with direct involvement in the destruction of intrahepatic bile ducts by autoreactive cytotoxic T cells [8–10]. Antigen specific $CD8^+$ T cells against PDC-E2 are enriched 10-fold in the liver compared to blood [11]. The chronic antigen stimulation of T cells has led to increased numbers of blood and liver highly differentiated $CD8^+$ T effector memory (TEM) cells identified as CD45RO + $CD8^+CD57^+$ [12] and $CD4^+$ T effector memory RA (TEMRA) cells identified as $CD4^+CD28^-$ T cells [13], with $CD8^+CD57^+$ T cells accumulating specifically around portal areas [12]. Both PBC blood and liver have increased $CD4^+CD28^-$cells. Antigen specific $CD4^+CD28^-$cells are cytotoxic and lyse biliary epithelial cells (BEC) in the absence of co-stimulation and do not become anergic [13].

Another marker of highly differentiated TEM and TEMRA cells is killer cell lectin-like receptor G1 (KLRG1). KLRG1 marks terminally differentiated T cells [14], like CD57, but its expression includes the less, but still highly, differentiated TEM population. In normal blood, approximately 95% $CD57^+$ T cells also express KLRG1 [15]. KLRG1 functions as an inhibitory receptor, and its ligands include E− and *N*-cadherins. The binding of E-cadherin to KLRG1 induces inhibitory signals in KLRG1+ T cells and NK cells, suppressing their activity [16].

Murine models of PBC have demonstrated a role of highly differentiated T cells [17]. In the NOD.c3c4 model, $CD8^+$ T cells, not $CD4^+$ T cells, are critical to passive transfer of disease and mediate direct bile duct injury [18]. In another model of PBC, dnTGFβRII mice deprived of regulatory TGF-β signaling restricted to T cells develop liver lesions similar to those of human PBC [19], indicative of T cells being the likely effectors of autoimmune cholangitis. Transfer of $CD8^+$ T cells, but not $CD4^+$ T cells, into Rag1 knockout recipients resulted in liver autoimmune disease that is histologically similar to PBC [20]. Large numbers of terminally differentiated $KLRG1^+$ CD8 T cells accumulate in the liver of dnTGFβRII mice [21].

To further understand the role of KLRG1+ cells in human PBC, here we examined the presence of KLRG1+ T cells in human liver biopsies and blood of patients with PBC and other immune related liver diseases, and the relationship of these cells to biomarkers of disease severity.

2. Methods

2.1. Gene expression data

Public domain gene expression data (Gene Expression Omnibus dataset GSE79850) from PBC (n = 16) and normal (n = 8) liver biopsies were analyzed for cytotoxic T cell differentiation status, with stratification according to risk of liver transplantation as previously described [22].

2.2. Patients

Liver samples and clinical data from patients with PBC (n = 44), autoimmune hepatitis (AIH) (n = 33), chronic hepatitis virus B (CHB) (n = 22), and healthy controls (HC) (n = 16), and blood samples from a separate cohort (PBC n = 16, AIH n = 14 and HC n = 15) were studied. Clinical features included age, gender, liver severity by hepatic inflammation stage and hepatic fibrosis stage [23], serum liver function tests, and anti-mitochondrial antibodies (Table 1). PBC, AIH and CHB patients was diagnosed according to established criteria [24–26]. Most PBC patients had stage 2 inflammatory degree and hepatic fibrosis; the proportions of patients in each inflammatory grade 1/2/3/4 was 16%/45%/39%/0 and in each fibrotic stage 0/1/2/3/4 was 5%/16%/52%/27%/2%, respectively.

All subjects provided written, informed consent prior to enrollment,

Table 1
Demographic and clinical features of patients with PBC, AIH, CHB, and HC performed immunohistochemistry in the study.

	HC (n = 16)	PBC (n = 44)	AIH (n = 33)	CHB (n = 22)
Age (years)	35.9 ± 6.5	42.3 ± 10.2	44.9 ± 12.7	41.4 ± 9.7
Gender (female/male)	8/8	33/11	25/8	9/13
ALT (U/L)	31.3 ± 20.8	71.6 ± 72.1	91.1 ± 88.7	73.2 ± 109.9
AST (U/L)	27.6 ± 15.1	60.0 ± 53.9	62.8 ± 51.0	50.9 ± 53.5
ALP (U/L)	95.7 ± 24.4	164.5 ± 131.5	125.7 ± 90.3	79.4 ± 23.9$^{\#}$
GGT (U/L)	42.5 ± 49.6	358.6 ± 186.3	88.3 ± 85.2*	47.2 ± 60.6$^{\#}$
TBIL (μmol/L)	16.5 ± 11.3	25.6 ± 41.2	30.3 ± 67.4	12.3 ± 3.7
DBIL (μmol/L)	5.6 ± 3.6	11.4 ± 25.7	18.7 ± 49.6	4.2 ± 1.3
IgM(g/L)	NA	2.8 ± 1.7	1.4 ± 0.6***	1.4 ± 0.8
IgG(g/L)	NA	14.6 ± 3.4	16.8 ± 6.1	15.1 ± 3.2
AMA(n)	NA	34	0	NA
Grade(inflammation, 1/2/3/4)	16/0/0/0	7/20/17/0	3/11/19/0	3/11/8/0
Stage(fibrosis, 0/1/2/3/4)	16/0/0/0/0	2/6/23/12/1	1/3/9/13/7	0/2/6/11/3

HC, healthy controls; CHB, chronic hepatitis B; PBC, primary biliary cholangitis; AIH, autoimmune hepatitis; ALT, alanine transaminase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, γ-glutamyl transferase; TBIL, total bilirubin; DBIL, direct bilirubin; IgM, immunoglobulin M; IgG, immunoglobulin G; AMA, anti-mitochondrial antibody; NA, not applicable; Continuous variables are expressed as mean ± standard error (SEM). $^{*}p < 0.05$ PBC vs AIH; $^{***}p < 0.001$ PBC vs AIH; $^{\#}p < 0.05$ PBC vs CHB.

and the study was approved by the Ethics Committee of Renji Hospital, Shanghai Jiao Tong University.

2.3. Immunohistochemistry (IHC) and confocal microscopy

Immunohistochemistry was performed on formalin-fixed, paraffin embedded liver samples from biopsies. Normal liver samples were obtained from patients undergoing hepatic hemangioma resection (n = 11) and liver donors for Orthotropic Liver Transplantation (n = 5). Although mild liver dysfunction was observed in five of the subjects (Table 1), none of these individuals had evidence of liver pathology. The protocol for IHC have been described previously. The liver sections were first deparaffinized and rehydrated, and then treated for heat mediated antigen retrieval with sodium citrate buffer (pH 6.0) for 15 min. Samples were then blocked with non-immune goat serum, followed by incubation with *anti*-KLRG1 antibody (1:100) (ABC-hyb008, Abcuro, Inc.) or *anti*-E-cadherin antibody (1:500) (EP700Y, Abcam) overnight at 4 °C. After washing in phosphate buffer saline (PBS), liver sections were incubated with horse radish peroxidase-conjugated anti-mouse/rabbit antibodies (Vector Laboratories) for 30 min at room temperature. Specific staining was detected by 3, 3′-diaminobenzidine (Vector Laboratories) and counted by light microscopy.Liver infiltration of KLRG1 + cells were assessed by counting absolute numbers of KLRG1 + cells per high power field (KLRG1 number) and the percentage of KLRG1 + cells among cells in portal lymphocytic infiltrates (KLRG1 score). KLRG1 scores were as follows: 0 = 0%, 1 = 0–25%, 2 = 26–50%, 3 = 51–75%, and 4 = 76–100%.

Confocal laser scanning microscopy was used to define the subtype of KLRG1 + cells infiltrated in portal areas in a subcohort of PBC patients (n = 7). The procedure was performed using our standard protocols [27]. In brief, after antigen retrieval, sections were blocked with 10% non-immune donkey serum for 30 min and then incubated with two primary antibodies, *anti*-KLRG1 (1:50) (ABC-hyb008, Abcuro, Inc.), and anti-CD4 (1:500) (EPR6855, Abcam), anti-CD8 (1:500) (EP1150Y, Abcam), or anti-CD56 (1:500) (EP2567Y, Abcam). Slides were incubated overnight at 4 °C, and then incubated with Alexa 488-conjugated donkey anti-mouse IgG (1:500) and Alexa 555-conjugated donkey anti-rabbit antibody (1:500) (Invitrogen/Life Technologies, UK) for 30 min at room temperature. The nucleus was stained by DAPI (SouthernBiotech, Birmingham, AL). Isotype controls were included for all immunostaining assays. Confocal scanning was performed using an LSM-710 laser-scanning confocal microscope (Carl Zeiss, Jena, Germany).

2.4. Flow cytometry

Multi-parametric flow cytometry was performed on frozen PBMCs. Briefly, thawed cells were first activated by Leukocyte Activaction Cocktail (BD Biosciences) in a 37 °C humidified CO2 incubator for 5 h, then stained cell surface markers with fluorochrome-conjugated monoclonal antibodies (mAbs), including APC-Cy7-*anti*-CD3 (Clone SK7, BD Biosciences), FITC-*anti*-CD3 (Clone UCHT1, BD Biosciences), FITC-*anti*-CD4 (Clone RPA-T4, Bio Legend), PE-cy7-*anti*-CD4 (Clone SK3, BD Biosciences), BV510-*anti*-CD8(Clone SK1, BD Biosciences),

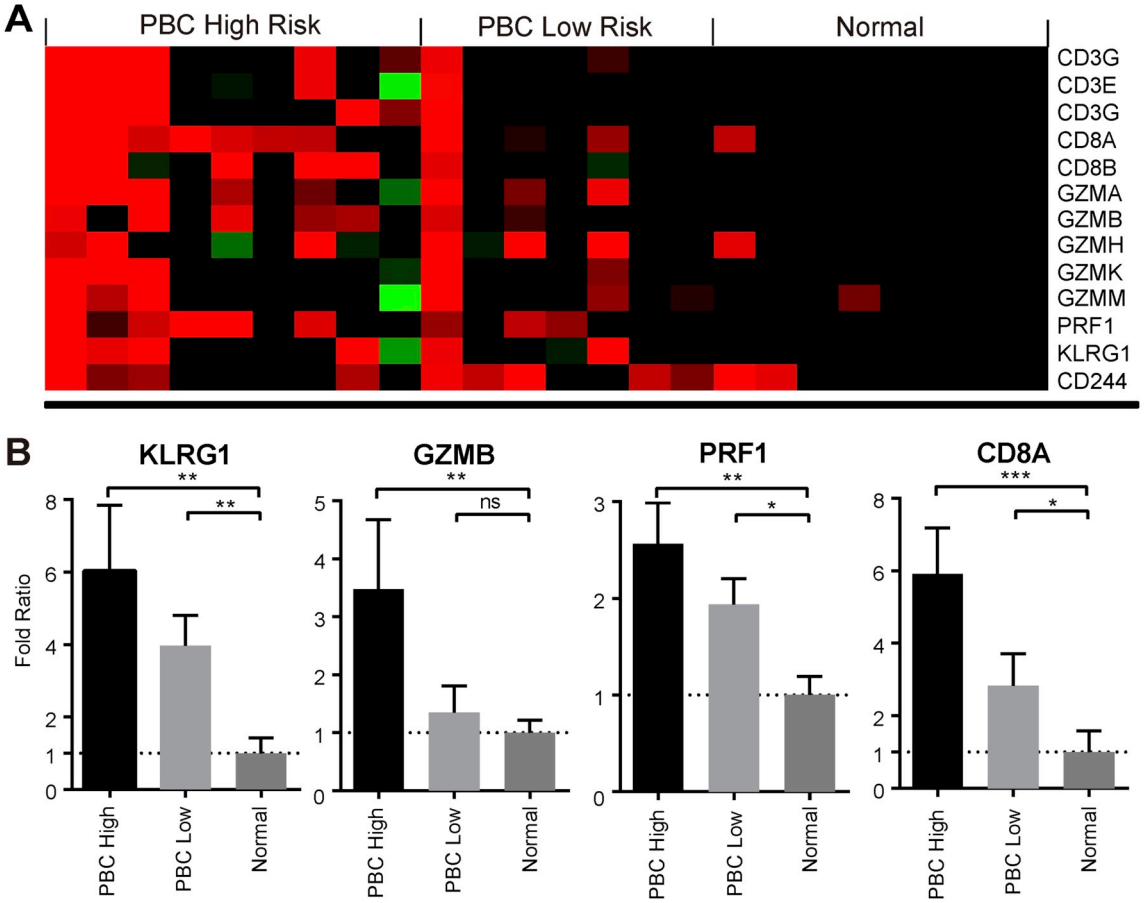

Fig. 1. T cell cytotoxicity and KLRG1 expression in PBC liver biopsies stratified by subsequent risk of liver transplantation. (A) Heatmap demonstrating T cell cytotoxic signature present in human PBC data (GSE79850) [19] stratified by high risk patients (those that required future liver transplant n = 9), low risk patients (those that remained responsive to UDCA and did not undergo future liver transplant n = 7), and normal (n = 8) liver biopsies. (B) Gene expression signature of highly differentiated $CD8^+$ cytotoxic T cells reflected in KLRG1, granzyme B (GZMB), perforin (PRF1), and CD8A expression stratified by future risk of liver transplantation. Mean and SEM shown. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

APC-*anti*-CD8(Clone RPA-T8, BD Biosciences), PE-*anti*-KLRG1 (Clone REA261, Miltenyi), AF700-labeled *anti*-CCR5 (Clone J418F1, Bio Legend), PerCP-Cy5.5-labeled *anti*-CX3CR1 (Clone 2A9-1 Bio Legend), then fixed and permeabilized with BD Cytofix/Cytoperm solution (BD Biosciences) for 20 min at 4 °C.Subsequently, intracellular staining was performed with AF647-labeled *anti*-Granzyme B(Clone GB11, BD Biosciences), BV421-*anti*-perforin (Clone δG9, BD Biosciences), AF700-labeled *anti*–IFN–γ(Clone B27, BD Biosciences), and PE-Cy7-*anti*-TNF (Clone MAb11, BD Biosciences). Phenotypic analysis of all samples was acquired on LSR Fortessa (BD Biosciences) and analyzed using FlowJo (BD Biosciences).

2.5. Enzyme-linked immunosorbent assay (ELISA)

E-cadherin levels in human plasma samples were detected using a human soluble E-cadherin ELISA kit (Abcam). A 40-fold dilution of these samples was performed before assaying.

2.6. Statistical analysis

Fold-ratios of Nanostring gene expression data (GSE79850) were analyzed in Graphpad Prism after normalization of genes to the normal samples. Mean and standard error of mean (SEM) values were plotted and *P*-values were calculated using nonparametric Mann-Whitney tests. Number and score of hepatic KLRG1 + cells were analyzed using non-parametric Mann-Whitney test or Kruskal-Wallis test, as appropriate. Correlations were performed using the Spearman's correlation coefficient. Wilcoxon matched-pairs signed rank test was used to compare levels of cytotoxic molecules, inflammatory cytokines and inflammatory chemokine receptors between KLRG1 + cells and KLRG1- counterparts. Expression of plasma soluble E-cadherin was analyzed using Student's *t*-test. All analyses were two-tailed and $p < 0.05$ was considered significant.

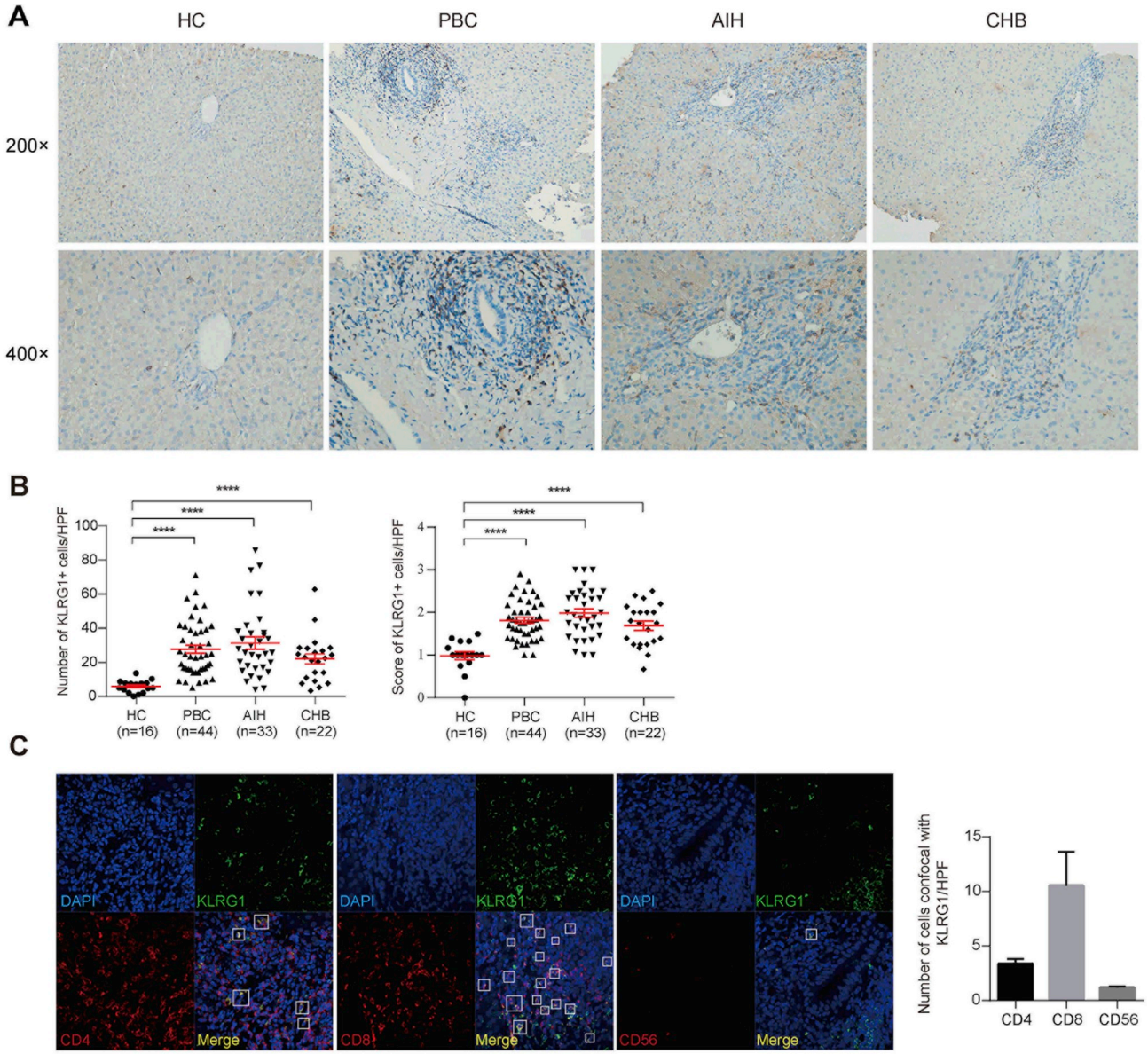

Fig. 2. KLRG1$^+$ lymphocytes, mostly CD8$^+$ T cells, are increased in PBC liver and invade portal tracts. (A) Representative immunohistochemistry image of KLRG1 in healthy controls (HC), primary biliary cholangitis (PBC), autoimmune hepatitis (AIH) and chronic hepatitis B (CHB) patients. (B) Statistical analysis of KLRG1 expression in HC (n = 16), PBC (n = 44), AIH (n = 33) and CHB (n = 22). (C) Representative double confocal staining of CD4 + KLRG1 +, CD8 + KLRG1 +, and CD56 + KLRG1 + cells using liver tissue of a subcohort of PBC patients (n = 7). Mean and SEM shown. $^{****}p < 0.0001$.

3. Results

3.1. Human PBC liver gene expression is characterized by a cytotoxic T cell signature and expression of KLRG1 correlated to long-term risk of liver transplantation

Previous analysis of liver gene expression data from patients with PBC identified a T cell activation signature associated with PBC that correlated with subsequent risk of liver transplantation [22]. Here, we analyzed these data for the presence of a cytotoxic T cell signature and expression of markers of highly differentiated T cells in PBC liver. We found a strong cytotoxic T cell signature and upregulation of KLRG1 (Fig. 1A). Stratification of data according to risk of liver transplantation showed that high risk patients had a higher cytotoxic T cell signature (e.g., granzyme B and perforin) and KLRG1 expression than low risk patients, both groups increased compared to normal (Fig. 1B).

3.2. KLRG1$^+$ T cells invade PBC portal tracts and their frequency correlated with liver inflammatory and fibrosis grades

Immunohistochemistry for KLRG1 demonstrated infiltration of PBC liver portal areas by KLRG1+ lymphocytes in 44/44 (100%) patients, with a mean (range) of KLRG1 scores of 1.8 (1–2.9) corresponding to approximately 25–75% of infiltrating cells (Fig. 2A and B). A significant increase of both absolute numbers of KLRG1+ cells per high power field and the score of KLRG1+ cells among cells in portal lymphocytic infiltrates were observed in biopsies from patients with PBC, AIH and CHB compared with healthy controls ($P < 0.0001$). We did not observe any difference in the frequency and score of KLRG1 between female and male patients with PBC. No significant difference was observed in the frequency and score of KLRG1 between HC with normal liver function and mild liver dysfunction. Immunofluorescence microscopy for double stained CD4+KLRG1+, CD8+KLRG1+, and CD56 + KLRG1+ cells in n = 7 patients demonstrated that the majority of KLRG1 cells infiltrating in portal tracts of PBC were CD8$^+$ T cells; 70% of KLRG1+ cells were CD8$^+$ T cells (10.5 ± 0.7), 22% were CD4$^+$ T cells (3.4 ± 0.2), and 8% were NK cells (1.2 ± 0.1) (Fig. 2C).

The degree of liver KLRG1+ lymphocyte infiltration as measured by the absolute number of KLRG1+ cells was positively correlated to both the hepatic inflammation score ($r = 0.47$, $p = 0.001$) and the hepatic fibrosis score ($r = 0.35$, $p = 0.021$) (Fig. 3A and B). The degree of KLRG1+ infiltration as score of inflammatory cells that are KLRG1+ was also positively correlated to the hepatic inflammation degree ($r = 0.42$, $p = 0.005$) (Fig. 3A). Additionally, although the correlation between score of hepatic KLRG1+ cells and hepatic fibrosis stage was not statistically significant, there was a trend of positive correlation ($r = 0.28$, $p = 0.065$) (Fig. 3B).

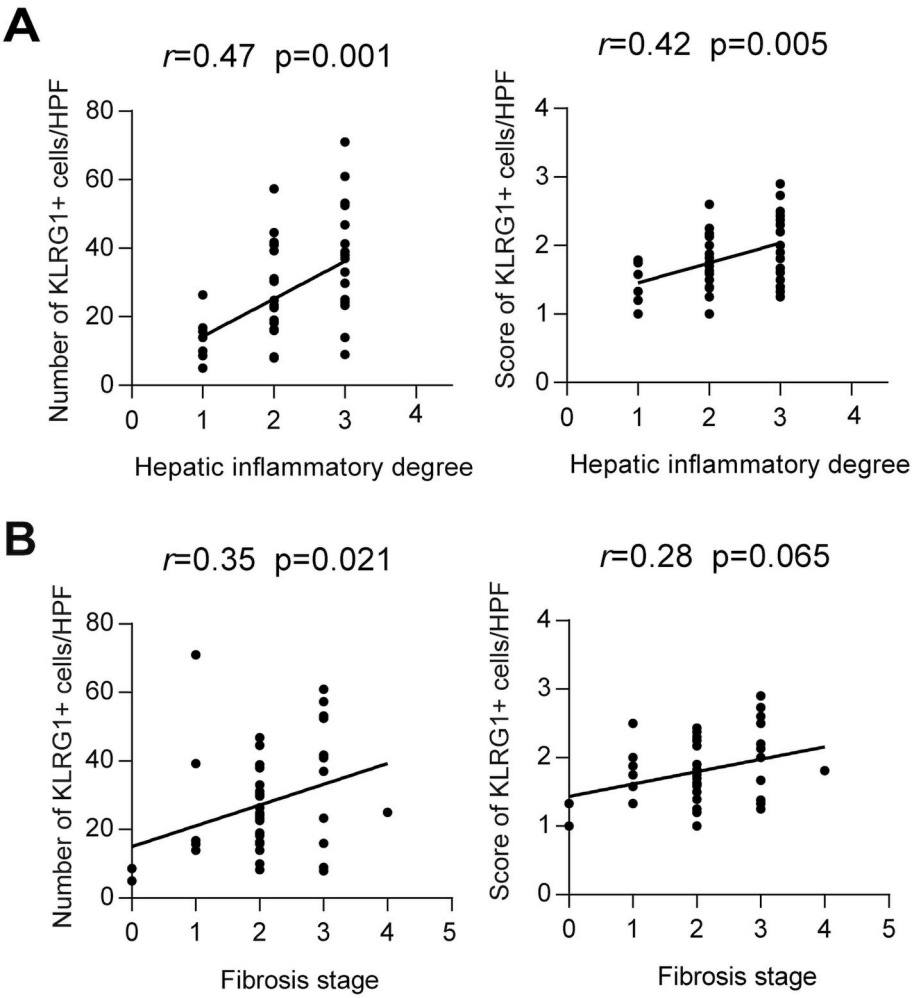

Fig. 3. PBC liver KLRG1$^+$ T cell infiltration is positively correlated with liver histology inflammatory and fibrosis scores (A) Absolute number and score of hepatic KLRG1+ cells were positively correlated to the hepatic inflammation score ($r = 0.47$, $p = 0.001$; $r = 0.42$, $p = 0.005$). (B) Frequency of hepatic KLRG1+ cells was positively correlated to the hepatic inflammation score ($r = 0.34$, $p = 0.02$); score of KLRG1+ cells had a trend of positive correlation to the hepatic fibrosis stage ($r = 0.28$, $p = 0.065$).

3.3. Liver KLRG1+ infiltration is positively correlated with disease activity biomarkers serum alkaline phosphatase and other liver function tests

Serum alkaline phosphatase (ALP) is an established surrogate biomarker of PBC disease activity [28]. We looked at the relationship of liver KLRG1+ lymphocyte infiltration to serum alkaline phosphatase. Among the PBC patients performed IHC in this study, hepatic KLRG1+ lymphocyte infiltration was positively correlated to serum ALP levels, measured by both absolute number of KLRG1+ lymphocytes ($r = 0.45$, $p = 0.005$) and score of KLRG1+ lymphocytes ($r = 0.43$, $p = 0.008$) (Fig. 4A). Serum GGT ($r = 0.40$, $p = 0.014$; $r = 0.32$, $p = 0.055$), ALT($r = 0.28$, $p = 0.085$; $r = 0.33$, $p = 0.047$), and AST ($r = 0.35$, $p = 0.033$; $r = 0.32$, $p = 0.047$) were similarly positively correlated to either absolute or score of KLRG1+ lymphocyte infiltration (Fig. 4B and Supplemental Figs. 1A and 1B). However, no correlation was observed between absolute or score KLRG1+ lymphocyte and serum total bilirubin and IgM levels (Supplemental Figs. 1C and 1D).

3.4. PBC blood KLRG1+ T cells are highly inflammatory cells enriched for cytotoxic molecules and inflammatory cytokines

We studied circulating KLRG1+ T cells in a separate cohort of 16 patients with PBC to characterize the potential role as inflammatory effector cells. In the $CD4^+$ T cell compartment, there was marked enrichment among CD4+KLRG1+ cells compared to CD4+KLRG1-cells for cells containing the cytotoxic molecules granzyme B (median: 51.40% [40.68–67.38%] vs median: 3.62% [2.14–6.78%], $p < 0.0001$)and perforin (median: 20.90% [13.28–23.40%] vs median: 6.60% [3.11–9.11%], $p < 0.0001$), and the inflammatory cytokines interferon gamma(IFN-γ) (median: 14.40% [8.03–18.75%] vs median: 4.15% [2.31–7.00%], $p < 0.0001$), and tumor necrosis factor alpha (TNF-α) (median: 10.07% [4.93–16.83%] vs median: 4.04% [1.97–6.35%], $p = 0.0002$) (Fig. 5A). Similarly, in the $CD8^+$ T cell compartment, there was marked enrichment among CD8+KLRG1+ cells compared to CD8+KLRG1-cells for cells containing granzyme B (median: 82.30% [73.38–88.75%] vs median: 21.30% [11.75–32.98%], $p < 0.0001$), perforin(median: 16.85% [13.13–28.95%] vs median: 8.24% [7.64–21.25%], $p = 0.029$), IFN-γ(median: 12.85% [7.89–20.90%] vs median: 8.15% [4.96–10.92%], $p = 0.003$), and TNF-α(median: 6.49% [2.08–9.44%] vs median: 2.44% [0.85–4.47%], $p = 0.004$) (Fig. 5B).

No significant difference was observed in the percentage of circulating CD4+KLRG1+ cells between HC, PBC, and AIH (Fig. 6A and B). However, circulating CD8+KLRG1+ lymphocytes were decreased in patients with PBC compared with HC (Fig. 6A and C). Blood cytotoxic (granzyme B and perforin) secreting CD4+KLRG1+ and $CD8^+$ KLRG1+cells were present in similar numbers in PBC and HC, yet the inflammatory cytokine secreting (KLRG1+IFN-γ+ and KLRG1+TNF-α+) KLRG1+ T cells were reduced in PBC compared to HC blood (Supplemental Figs. 2A and 2B).

We then detected the expression of inflammatory chemokine receptors CCR5, and CX3CR1 in KLRG1+ and KLRG1-lymphocytes of PBC patients. Interestingly, we found that KLRG1+ lymphocytes from PBC blood express increased levels of CX3CR1 and CCR5 than their

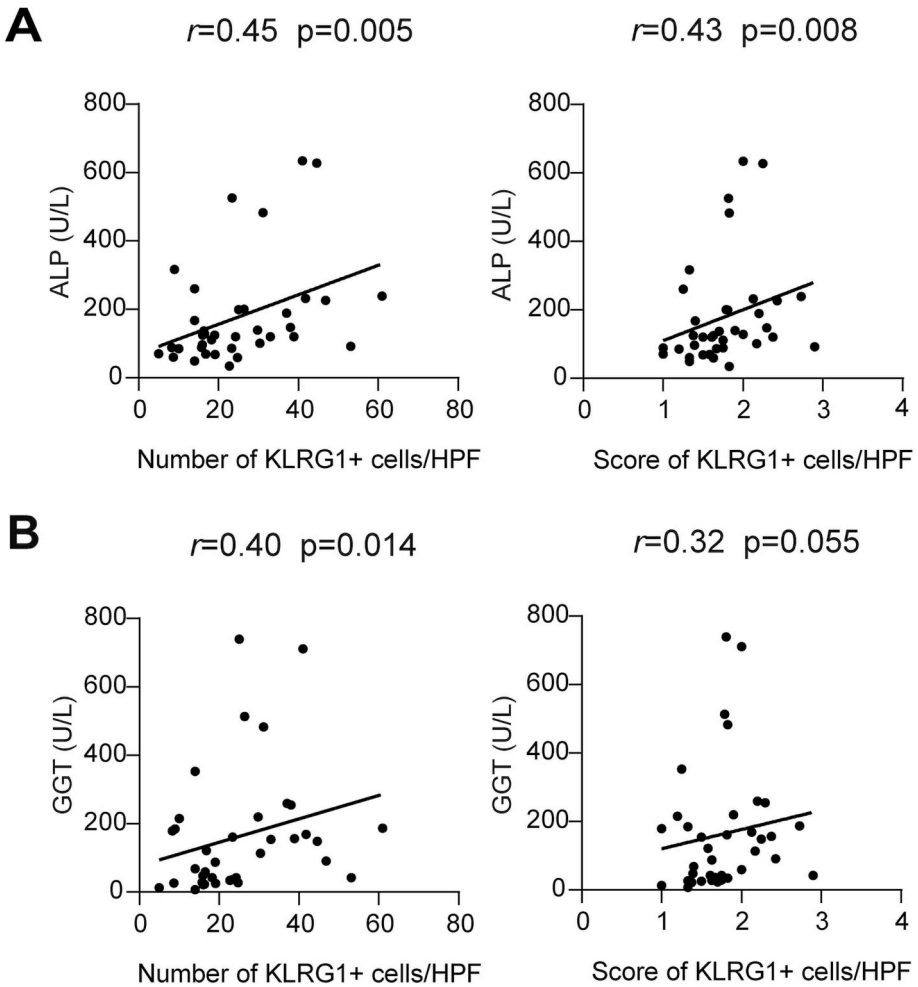

Fig. 4. Liver KLRG1+ infiltration is positively correlated with serum disease activity biomarkers Absolute number or score of KLRG1+ lymphocyte was positively correlated with serum ALP (A), GGT (B) in patients with PBC.

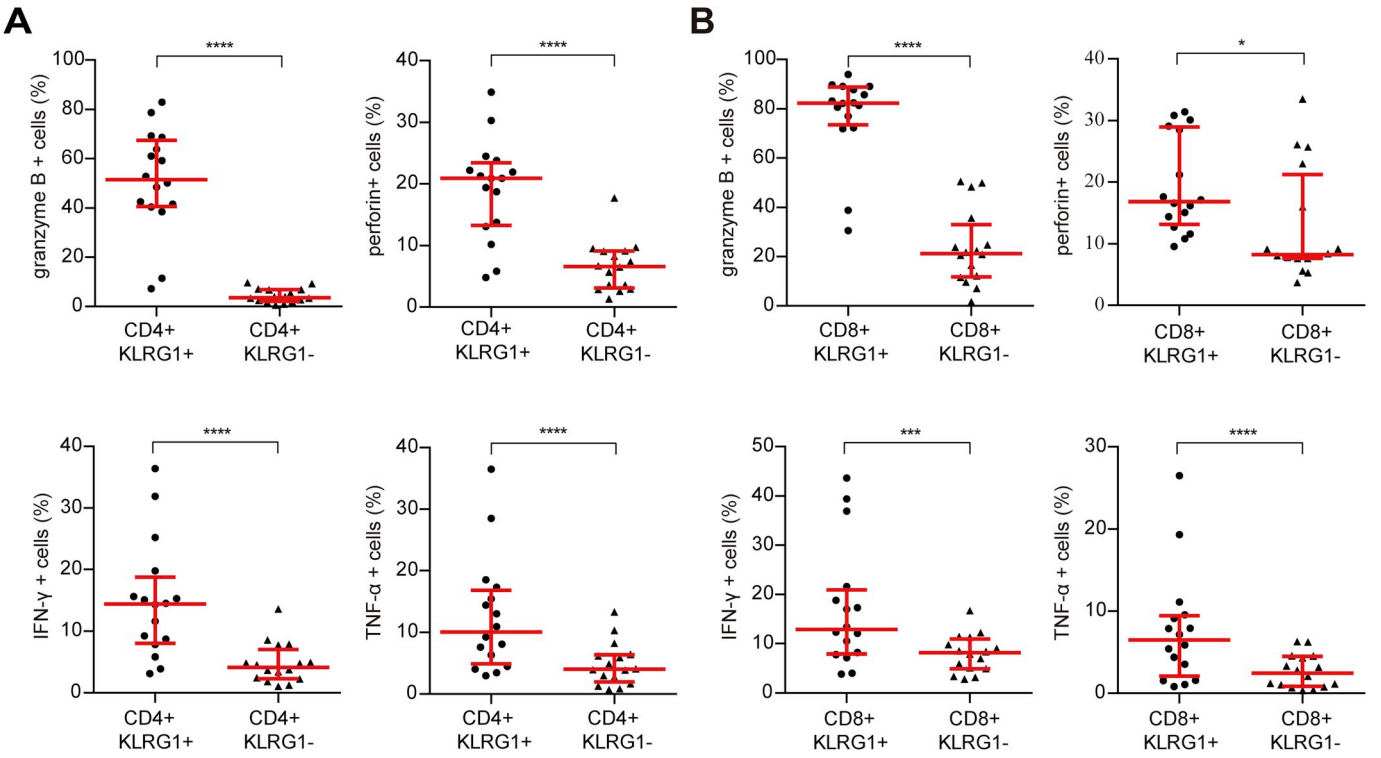

Fig. 5. PBC circulating KLRG1 + T cells are highly cytotoxic inflammatory cells. Increased cytotoxic molecules (granzyme B and perforin), and inflammatory cytokines (IFN-γ and TNF-α) were observed in KLRG1 + lymphocytes compared to KLRG1-counterparts in both (A) $CD4^+$ T cell and (B) $CD8^+$ T cell populations. Median and interquartile range (IQR) shown. $^*p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

KLRG1-counterparts (Fig. 6D and E). Together with the liver histology findings of increased PBC liver KLRG1 + cells (Fig. 2B and C), these data suggest that in PBC, circulating inflammatory KLRG1 + T cells, especially CD8 + KLRG1 + lymphocytes, are retained and enriched in the portal tracts.

3.5. The inhibitory ligand for KLRG1, E-cadherin, partially disappears in intrahepatic small bile ducts of PBC patients

To further understand the susceptibility of the portal areas to KLRG1 + T cell inflammation, we examined the distribution of E-cadherin in liver, as E-cadherin is a major inhibitory ligand for KLRG1 [16]. By immunohistochemistry, we found that a clear membranous pattern of E-cadherin was expressed in hepatocytes and BEC in normal human liver, as previously reported [29,30]. However, in PBC patients, there was partial loss, with only fragmented expression in 75% of intrahepatic small bile ducts (15/20), suggesting a potential mechanism for the increased susceptibility of these portal areas to infiltration by KLRG1 + lymphocytes (Fig. 7A). Soluble E-cadherin may also be shed from E-cadherin expressing cells and has been postulated to be a mechanism of natural defense against KLRG1 + cells [31]. Intriguingly, we found by ELISA an increase in plasma soluble E-cadherin in PBC compared to healthy controls (85.92 ± 93.55 vs 63.69 ± 32.38, $p = 0.044$) (Fig. 7B).

4. Discussion

The pathogenesis of PBC is complex and involves multiple arms of the immune system [32,33]. Since the identification of PDC-E2 as the target of B cell produced anti-mitochondrial antibodies (AMA) [34], it has become clear that the binding targets of AMA and $CD4^+$ T and $CD8^+$ T cell antigen receptors [11] are all located in the same region of BEC expressed PDC-E2 [35]. Involvement of T cells is a major feature in the pathogenesis of PBC [8–13]. In particular, the chronic antigen stimulation of T cells has led to increased numbers of blood and liver highly differentiated $CD4^+$ and $CD8^+$ TEM and TEMRA cells. Because KLRG1 is a known marker of highly differentiated cytotoxic T cells, we studied the potential role of KLRG1 + lymphocytes in mediating PBC.

We identified large numbers of KLRG1 + infiltrating lymphocytes, including $CD4^+$, $CD8^+$, and NK cells, present in PBC liver portal areas, accounting for roughly 25–75% of infiltrating cells. The abundance of these cells correlates with both histological hepatic inflammation severity and histologic hepatic fibrosis severity. In PBC blood, these cells contain substantially more cytotoxic molecules (granzyme B and perforin) and inflammatory cytokines (IFN-γ and TNF-α) than their KLRG1-counterparts. Moreover, these cells express significantly higher levels of inflammatory chemokine receptors (CCR5 and CX3CR1). CCR5 participates in recruiting lymphocytes to portal areas [36,37], whereas CX3CR1 could be recruited by injured BEC through the overexpression of chemokines CX3CL1 in PBC [38]. This leads us to propose that cytotoxic KLRG1 + cells use CCR5 to enter the portal tracts and then localize to damaged BEC by CX3CR1. Interestingly, circulating CD8 + KLRG1 + were decreased in PBC patients compared with HC, consistent with the fact that the majority of KLRG1 + infiltrating lymphocytes were $CD8^+$ T cells. These findings suggest that inflammatory KLRG1 + lymphocytes, especially cytotoxic $CD8^+$ KLRG1 + T cells, reduced in circulation but accumulating around inflamed bile ducts, may have a major role in the injury of BEC in PBC. Additionally, these studies suggest that KLRG1 positive lymphocytes may play a role in autoimmune hepatitis as well and that further study is warranted.

We additionally examined E-cadherin, a ligand for KLRG1 inhibitory signaling [16], in human liver. Previous studies in mice have suggested that BEC expression of E-cadherin suppressed peri-portal inflammation, as mouse conditional BEC E-cadherin knockout resulted in cholangitis [29,30]. These and other studies have considered a potential contributing mechanism of cholangitis to relate to E-cadherin's function in maintaining epithelial tight junctions, which is essential for maintaining normal liver functions [39]. The current studies suggest

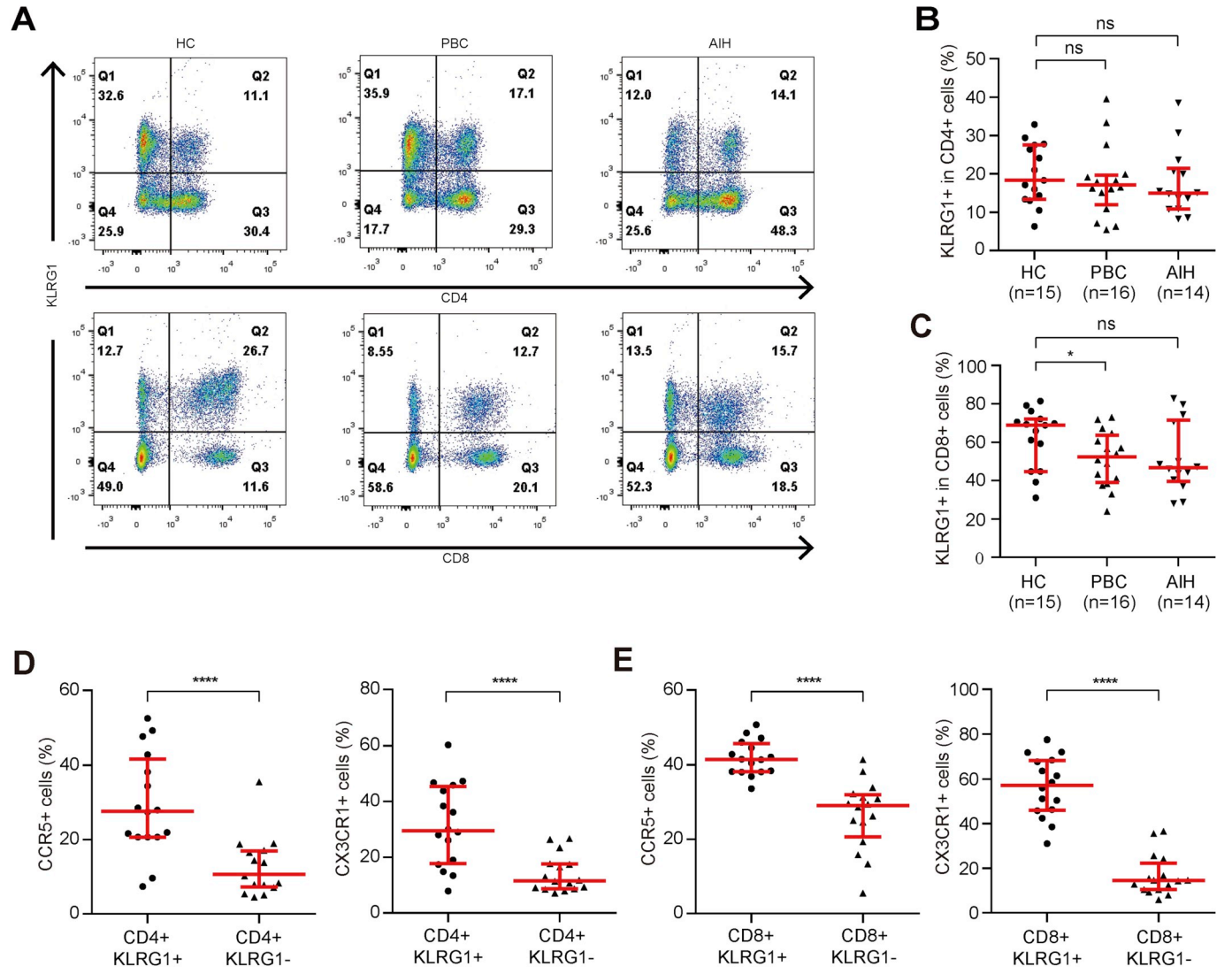

Fig. 6. Decreased proportion of cytotoxic and inflammatory cytokine secreting KLRG1 + cells in PBC blood suggest their enrichment in liver. (A) Representative scatter plots of $CD4^+$ KLRG1 + and CD8 + KLRG1 + cells from a HC and representative patient with PBC, AIH. (B) No significant difference was observed in the percentage of circulating CD4 + KLRG1 + cells between HC, PBC, and AIH. (C) Decreased circulating CD8 + KLRG1 + were observed in PBC patients compared with HC. Significantly increased levels of CCR5 and CX3CR1 were observed in KLRG1 + lymphocytes compared to KLRG1-counterparts in both (D) $CD4^+$ T cell and (E) $CD8^+$ T cell populations. Median and interquartile range (IQR) shown. ns, not significant, $^*p < 0.05$, $^{****}p < 0.0001$.

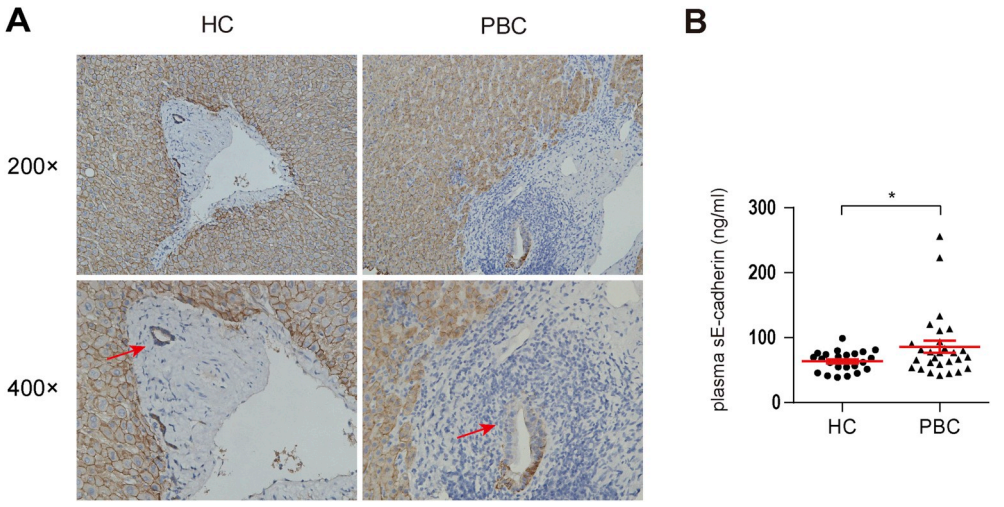

Fig. 7. Lack of expression of E-cadherin, an inhibitory of KLRG1 + lymphocytes, in intrahepatic small bile ducts of PBC patients (A) Representative immunohistochemistry image of hepatic E-cadherin expression in human normal liver and a representative patient with PBC. A clear membranous pattern of E-cadherin expression in the epithelial cells of intrahepatic small bile ducts was seen in normal liver (n = 10), whereas it partially disappeared, with only fragmented expression in fifteen of twenty PBC samples. (B) Plasma levels of sE-cadherin were increased in patients with PBC compared to in HC. Mean and SEM shown. $^*p < 0.05$.

that an additional mechanism of peri-portal cholangitis in association with decreased E-cadherin expression could relate to decreased inhibition of inflammatory KLRG1+ lymphocytes.

To understand the relationship between liver portal area KLRG1+ cells and PBC disease severity, we correlated liver KLRG1 measurements to two measures of clinical disease severity, future need for liver transplantation and serum ALP. We found that KLRG1 gene expression in PBC liver was positively correlated to risk of liver transplantation in patients followed for at least 15 years after liver biopsy. We then directly demonstrated that KLRG1+ lymphocyte infiltration occurred in PBC portal areas and the degree of this infiltration was positively correlated with the surrogate endpoint of serum ALP, an outcome measure that has been accepted by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) as a surrogate outcome measure for conditional registration of obeticholic acid for PBC [28].

These results link presence of KLRG1+ lymphocytes to clinical outcome measures and suggest that the autoimmune attack in PBC may be driven by KLRG1 bearing cytotoxic late differentiated T cells. Therapeutic approaches that modulate KLRG1+ T cells, such as specific depletion of KLRG1 expressing lymphocytes, would represent a rational new strategy for treatment of PBC.

Author contributions

XM, SAG, MEG and RT designed and supervised the project. XM, RT JF and QW obtained the funding. YL contributed to acquisition of data, analysis of data and drafting of the manuscript. BL and ZY performed interpretation of data and drafting of the manuscript. SAG performed drafting of the manuscript. JZ contributed to analysis of data. YW, YL, YC and BH collected samples. XM and QW performed clinical diagnosis and treatment. QM and YP provided technical support. XM, MEG and RT revised the manuscript for important intellectual content.

Conflicts of interest

SAG is a founder of Abcuro, Inc., which is developing KLRG1 targeted therapies. M.E.G. is a consultant to Abcuro, Inc. The other authors disclose no other conflicts.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2019.06.004.

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